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The double-stranded RNA (dsRNA) analogue poly(I:C) is a promising adjuvant for cancer vaccines because it activates both dendritic cells (DCs) and natural killer (NK) cells, concurrently promoting adaptive and innate anticancer responses. Poly(I:C) acts through two dsRNA sensors, Toll-like receptor 3 (TLR3) and melanoma differentiation-associated protein-5 (MDA5). Here, we investigated the relative contributions of MDA5 and TLR3 to poly(I:C)-mediated NK cell activation using MDA5$^{-/-}$, TLR3$^{-/-}$, and MDA5$^{-/-}$TLR3$^{-/-}$ mice. MDA5 was crucial for NK cell activation, whereas TLR3 had a minor impact most evident in the absence of MDA5. MDA5 and TLR3 activated NK cells indirectly through accessory cells and induced the distinct stimulatory cytokines interferon-α and interleukin-12, respectively. To identify the relevant accessory cells in vivo, we generated bone marrow chimeras between either wild-type (WT) and MDA5$^{-/-}$ or WT and TLR3$^{-/-}$ mice. Interestingly, multiple accessory cells were implicated, with MDA5 acting primarily in stromal cells and TLR3 predominantly in hematopoietic cells. Furthermore, poly(I:C)-mediated NK cell activation was not notably impaired in mice lacking CD8α DCs, providing further evidence that poly(I:C) acts through diverse accessory cells rather than solely through DCs. These results demonstrate distinct yet complementary roles for MDA5 and TLR3 in poly(I:C)-mediated NK cell activation.

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Abbreviations used: DKO, double KO; dsRNA, double-stranded RNA; IFNAR, IFN-α/β receptor.

Microbial components play a major role in activating innate and adaptive immune responses by triggering pattern recognition receptors (Ishii et al., 2008). Poly(I:C) is an analogue of viral double-stranded RNA (dsRNA) that activates various immune cell types through two major dsRNA sensors, melanoma differentiation-associated protein-5 (MDA5) and Toll-like receptor 3 (TLR3). MDA5 is a cytosolic sensor, which detects poly(I:C) that penetrates into the cytosol through as yet undefined mechanisms (Ishii et al., 2008). TLR3 is located in intracellular endosomes and detects poly(I:C) that has been internalized by endocytosis (Matsumoto and Seya, 2008). Upon poly(I:C) detection, MDA5 transmits signals through the adaptor IPS1, whereas TLR3 signals through the adaptor TRIF (also known as TICAM1). Both of these adaptors initiate downstream signaling pathways that lead to activation of a similar array of transcription factors, including IRF3, IRF7, IRF1, and NF-κB. These factors induce the expression of genes encoding type I IFNs: i.e., IFN-α and IFN-β, proinflammatory cytokines, and various molecules involved in antigen presentation (Kawai and Akira, 2008).

Poly(I:C) induces the maturation of DCs, boosting their ability to prime and expand antigen-specific T cell responses (Kumar et al., 2008; Trumpfheller et al., 2008; Longhi et al., 2009). Because of this DC stimulatory activity, poly(I:C) is a promising adjuvant for vaccines, particularly for cancer vaccines that must overcome both tolerance to tumor-associated self-antigens and the immunosuppressive influence

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of the tumor microenvironment (Steinman and Banchereau, 2007). Poly(I:C) is also extensively used to activate mouse NK cells in vivo. The NK cell stimulatory activity of poly(I:C) is potentially important for anticancer vaccines, as it may contribute to tumor eradication by inducing NK cell–mediated lysis of tumor cells. In humans, the NK cell stimulatory activity of poly(I:C) has been chiefly attributed to its ability to trigger TLR3 expressed in cultured NK cells (Schmidt et al., 2004; Sivori et al., 2004; Hart et al., 2005; Lauzon et al., 2006). Whether poly(I:C) also activates human NK cells through DCs or other accessory cells has not been investigated. In mice, poly(I:C) is thought to activate NK cells primarily through DCs. An initial study showed that poly(I:C) stimulates TLR3 in DCs, which consequently acquire the ability to activate NK cells (Akazawa et al., 2007). A very recent study showed that poly(I:C) triggers both the TRIF and IPS1 signaling pathways in CD8α DCs, which in turn activate NK cells in vitro (Miyake et al., 2009). These results suggest that TLR3 and MDA5 may stimulate mouse NK cells indirectly through activation of DCs, particularly CD8α DCs.

In this study, we investigate the relative contributions of MDA5 and TLR3 in poly(I:C)-mediated activation of NK cells using MDA5$^{-/-}$, TLR3$^{-/-}$, and MDA5$^{-/-}$TLR3$^{-/-}$ mice. We find that MDA5 has a predominant role in NK cell activation, whereas the contribution of TLR3 is secondary and is most evident in the absence of MDA5. Both MDA5 and TLR3 activated NK cells indirectly through accessory cells but induced different NK cell stimulatory cytokines, as MDA5 was essential for IFN-α and IFN-β, whereas TLR3 was required for IL-12 and, in part, for IFN-β. By generating BM chimeras between WT and dsRNA sensor-deficient mice, we found that MDA5 promotes NK cell activation mainly through stromal accessory cells, whereas TLR3 acts predominantly through BM-derived accessory cells. To determine whether the hematopoietic accessory cells that activate NK cells are indeed CD8α DCs, we examined mice deficient for the basic leucine zipper transcription factor ATF-like 3 (BATF3), which have a selective developmental defect in CD8α DCs (Hildner et al., 2008). We found that poly(I:C)-mediated stimulation of NK cells was minimally affected in these mice, indicating that poly(I:C) acts through multiple accessory cells rather than solely through CD8α DCs. We conclude that MDA5 and TLR3 mediate substantially distinct and yet complementary functions during poly(I:C)-mediated activation of NK cells.

RESULTS AND DISCUSSION

MDA5 has a predominant role in the NK cell response to poly(I:C) in vivo

To investigate the relative contributions of MDA5 and TLR3 in poly(I:C)-mediated activation of NK cells, we injected WT, MDA5$^{-/-}$, TLR3$^{-/-}$, and MDA5$^{-/-}$TLR3$^{-/-}$ (double KO [DKO]) mice with poly(I:C); after 24 h, we isolated spleen NK cells and measured cytotoxicity ex vivo. NK cells from untreated WT and sensor-deficient mice were unable to kill targets, whereas NK cells from poly(I:C)-treated WT mice killed up to 50% of targets (Fig. 1 A). NK cells from MDA5$^{-/-}$ mice treated with poly(I:C) had a significant defect in cytotoxicity, as only 20% of target cells were killed at maximum effector/target ratios. NK cells from TLR3$^{-/-}$ mice treated with poly(I:C) had a modest but not significant cytotoxicity defect. However, NK cells from DKO mice were unable to lyse targets after poly(I:C) stimulation. These results suggest that MDA5 can largely compensate for the lack of TLR3 and that TLR3 contribution to NK activation is evident only in the absence of MDA5.

Another measure of NK cell activation is CD69 upregulation. CD69 is a cell surface molecule induced by IFN-α/β that promotes lymphocyte retention in lymphoid

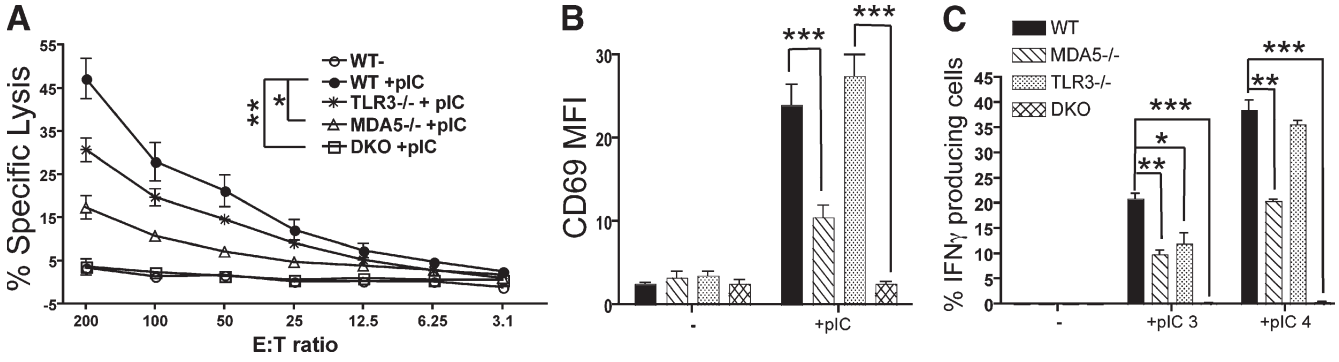

Figure 1. Poly(I:C)-induced NK cell activation is primarily mediated by MDA5. WT, MDA5$^{-/-}$, TLR3$^{-/-}$, and DKO mice were injected with 100 µg poly(I:C) i.v. After 24 h, splenocytes were harvested and used as effector cells in a cytotoxicity assay with labeled RMA-S targets (experiment performed using one mouse for each genotype in four independent trials; A) or assayed for CD69 expression by FACS, gating on NK1.1$^+$CD3$^-$ splenocytes (experiment performed using two mice for each genotype in three independent trials; B). Alternatively, 3 or 4 h after poly(I:C) injection, splenocytes were isolated, cultured with monensin for an additional 3 or 4 h, and analyzed for intracellular content of IFN-γ by FACS, gating on NK1.1$^+$CD3$^-$ cells (experiment performed using one mouse for each genotype in three independent trials; C). Statistical significance is indicated by *, $P < 0.05$, **, $P < 0.001$, and ***, $P < 0.0001$. Error bars represent SD.

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organs (Shiow et al., 2006). After injection of poly(I:C), CD69 up-regulation was partially impaired in NK cells from MDA5$^{-/-}$ mice in comparison with those from WT mice and was completely abrogated in NK cells from DKO mice (Fig. 1 B). There was no decrease in CD69 expression in the TLR3$^{-/-}$ mice, further implying that the effect of poly(I:C) is predominantly mediated by MDA5. To determine the contributions of MDA5 and TLR3 to NK cell production of IFN-γ in response to poly(I:C), we isolated splenocytes 3 and 4 h after injecting WT and dsRNA sensor-deficient mice with poly(I:C) and determined the intracellular content of IFN-γ in NK cells. NK cells isolated from both MDA5$^{-/-}$ and TLR3$^{-/-}$ mice 3 h after poly(I:C) injection produced less IFN-γ than WT NK cells (Fig. 1 C). However, by 4 h after poly(I:C) injection, TLR3$^{-/-}$ and WT NK cells generated similar amounts of IFN-γ, whereas MDA5$^{-/-}$ NK cells still produced less IFN-γ than either the TLR3$^{-/-}$ or WT NK cells. DKO NK cells did not produce IFN-γ at any time point assessed after poly(I:C) stimulation. All together, these results indicate that MDA5 plays a more predominant role than TLR3 in stimulating NK cytotoxicity, CD69 up-regulation, and IFN-γ production.

MDA5 activates NK cells through an NK cell–extrinsic pathway

Because MDA5 is ubiquitously expressed, poly(I:C) could directly activate mouse NK cells through MDA5. However, MDA5 is also expressed in DCs, which play a crucial role in activating NK cells (Fernandez et al., 1999; Ferlazzo et al., 2002; Gerosa et al., 2002; Andrews et al., 2003; Andoniou et al., 2005; Lucas et al., 2007; Mortier et al., 2008). Thus, poly(I:C) may activate mouse NK cells through DCs or other accessory cells expressing MDA5. To test whether MDA5-mediated activation of mouse NK cells occurs in an NK-intrinsic or -extrinsic manner, we co-cultured combinations of BMDCs and NK cells from WT or dsRNA sensor-deficient mice and measured cytotoxicity, CD69 up-regulation, and IFN-γ production in response to poly(I:C). Remarkably, the defect seen in NK cell activation in the MDA5$^{-/-}$ mice in vivo was entirely recapitulated in the co-cultures of WT NK cells with MDA5$^{-/-}$ BMDCs. MDA5$^{-/-}$ BMDCs stimulated with poly(I:C) promoted NK cytotoxicity, CD69 up-regulation, and IFN-γ secretion less effectively than did poly(I:C)-activated WT BMDCs (Fig. 2, A–C). After exposure to poly(I:C), DKO BMDCs were almost entirely incapable of inducing NK activation. TLR3$^{-/-}$ BMDCs stimulated with

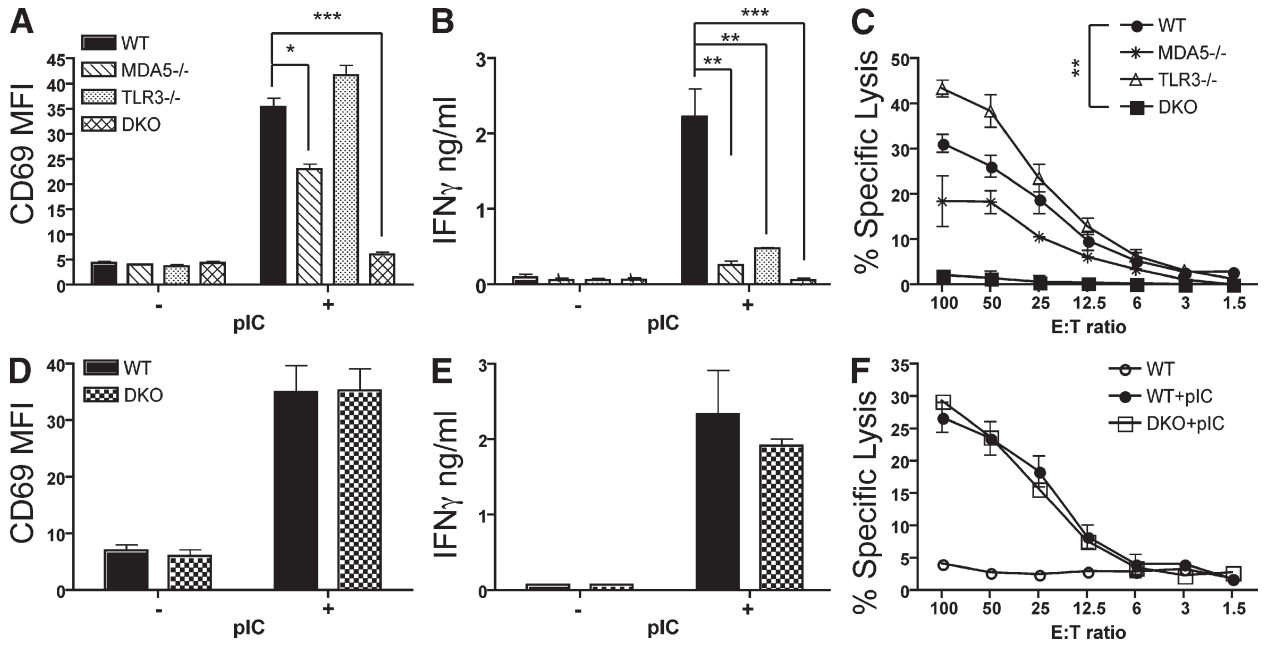

Figure 2. MDA5 and TLR3 activate NK cells through NK cell–extrinsic mechanisms. BMDCs from WT, MDA5$^{-/-}$, TLR3$^{-/-}$, and DKO mice were cultured with purified NK cells from WT mice in the presence or absence of 25 µg poly(I:C). After 24 h, NK1.1^{+}CD3^{-} cells were stained for CD69 expression (A), and IFN-γ was measured from cultured supernatants (experiments were performed using BMDCs from one mouse for each genotype and NK cells from three pooled mice in four independent trials; B). Alternatively, Cr^{51}-labeled RMA-S targets were added to culture, and cytotoxicity was measured (experiments were performed using BMDCs from one mouse from each genotype and NK cells from three pooled mice in three independent trials; C). In reverse experiments, BMDCs from WT mice were cultured with purified NK cells from WT or DKO mice with or without poly(I:C). After 24 h, CD69 expression was determined by FACS (D), IFN-γ was measured in supernatants (experiments were performed using BMDCs from one mouse and NK cells from three pooled mice of each genotype in four independent trials; E), and cytotoxicity was measured against RMA-S targets (experiments were performed using BMDCs from one mouse and NK cells from three pooled mice of each genotype in three independent trials; F). Statistical significance is indicated by *, $P < 0.05$, **, $P < 0.001$, and ***, $P < 0.0001$. Error bars represent SD.

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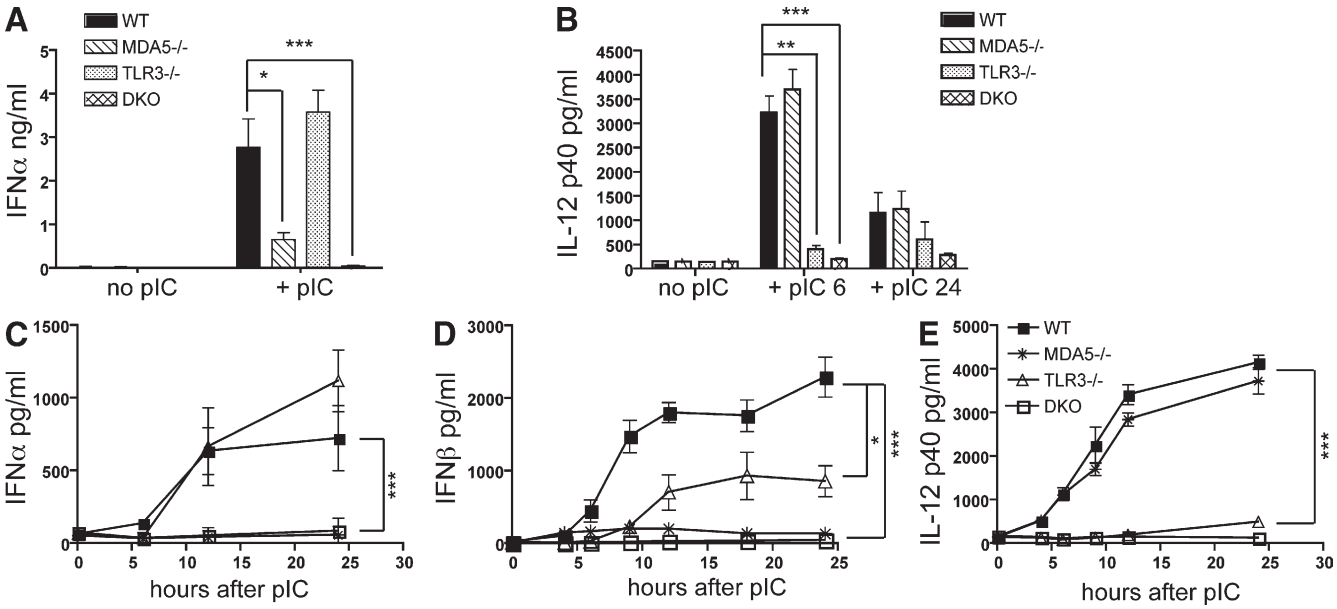

Figure 3. MDA5 and TLR3 mediate distinct cytokine responses. WT, MDA5$^{-/-}$, TLR3$^{-/-}$, and DKO mice were injected with 100 µg poly(I:C) i.v. Serum was taken at 6 and 24 h and assayed for IFN-α (experiment was performed with serum from five mice of each genotype in one independent ELISA assay; A) or IL-12p40 (experiment was performed with serum from four mice of each genotype in one independent ELISA assay; B) by ELISA. BMDCs from WT, MDA5$^{-/-}$, TLR3$^{-/-}$, and DKO mice were stimulated with 25 µg poly(I:C). At various time points after stimulation, supernatants were harvested, and IFN-α (C), IFN-β (D), and IL-12p40 (E) were measured by ELISA (experiments were performed with supernatants from BMDCs from each genotype in four independent experiments and evaluated by two independent ELISA assays). Statistical significance is indicated by *, $P < 0.05$, **, $P < 0.001$, and ***, $P < 0.0001$. Error bars represent SD.

poly(I:C) induced less IFN-γ production in NK cells than did similarly treated WT BMDCs, whereas NK cell cytotoxicity and CD69 expression were slightly augmented. No significant differences in NK cytotoxicity, CD69 expression, and IFN-γ production were detected when NK cells from WT or DKO mice were co-cultured with poly(I:C)-activated WT BMDCs (Fig. 2, D–F). Consistent with this result, purified NK cells exhibited only modest or no increase in CD69 expression and IFN-γ secretion when directly stimulated with poly(I:C), even when pretreated with IFN-α and/or IL-12 to induce MDA5 and TLR3 (Fig. S1). We conclude that poly(I:C)-induced NK activation through MDA5 and TLR3 occurs extrinsic to the mouse NK cell itself. Moreover, although MDA5 deficiency in BMDCs severely impaired cytotoxicity and CD69 expression, TLR3 deficiency had a minor impact on these functions. In fact, TLR3 deficiency caused a slight increase of cytotoxicity and CD69 expression. These in vitro results further corroborate the concept that MDA5 plays a predominant role in mouse NK cell activation, whereas the contribution of TLR3 is limited but quite evident in the complete abrogation of NK activation observed in the DKO mice and cells.

MDA5 and TLR3 disparately promote the secretion of cytokines that stimulate NK cells

A variety of cytokines has been shown to activate NK cells. IFN-α/β augments NK cell lytic capacity and expression of CD69 (Gerosa et al., 2002, 2005; Swann et al., 2007); IFN-α/β, IL-12, and IL-18 stimulate NK cell production of IFN-γ (Trinchieri, 1995; Biron et al., 1999; Nguyen et al., 2002; Ferlazzo and Münz, 2004; Andoniou et al., 2005; Chaix et al., 2008); and IL-15 and IL-2 promote NK cell survival, proliferation, and effector functions (Waldmann and Tagaya, 1999; Granucci et al., 2004; Koka et al., 2004; Lucas et al., 2007; Mortier et al., 2008). Because stimulation of both MDA5 and TLR3 with poly(I:C) leads to the production of IFN-α/β as well as inflammatory cytokines in DCs and other cells (Kawai and Akira, 2008), we predicted that the defect in NK activation in vivo would be associated with a defect in cytokine production in the absence of these dsRNA sensors. We found that serum IFN-α was completely abolished in the MDA5$^{-/-}$ and DKO mice 24 h after poly(I:C) stimulation (Fig. 3 A). In contrast, there was no defect in serum IFN-α in TLR3$^{-/-}$ mice compared with WT mice, which is consistent with previous studies (Kato et al., 2006; Kumar et al., 2008; Miyake et al., 2009). MDA5$^{-/-}$ mice had WT levels of IL-12p40 in the serum, whereas serum IL-12p40 was completely abolished in TLR3$^{-/-}$ and DKO serum 6 h after poly(I:C) stimulation (Fig. 3 B). Other cytokines potentially relevant for NK cell activation, such as IFN-β, IL-18, IL-1β, IL-15, or IL-12p70, were undetectable in the serum of all mice after poly(I:C) stimulation.

Similar to our findings in the serum, we found that poly(I:C)-stimulated MDA5$^{-/-}$ and DKO BMDCs secreted less IFN-α than did WT BMDCs (Fig. 3 C). TLR3 deficiency did not diminish but, in fact, slightly augmented the IFN-α response.

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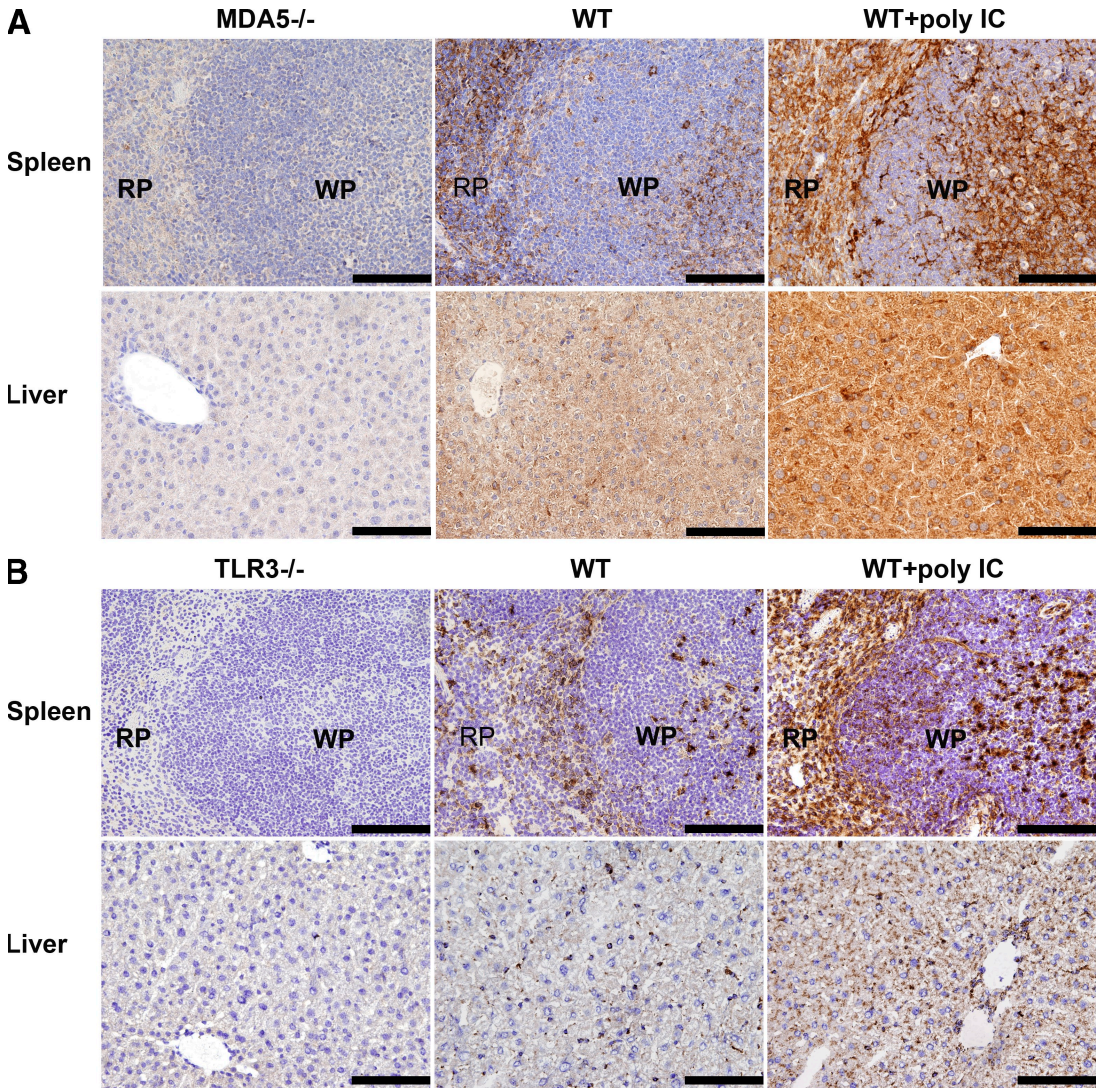

Figure 4. Expression patterns of MDA5 and TLR3 in spleen and liver. (A) Frozen tissue sections from spleen and liver of unstimulated MDA5$^{-/-}$ and WT mice and from poly(I:C)-injected WT mice were stained with anti-MDA5 (brown) and counterstained with hematoxylin (blue). (B) Formalin-fixed sections from spleen and liver of unstimulated TLR3$^{-/-}$ and WT mice and poly(I:C)-injected WT mice were stained with anti-TLR3 (brown) and counterstained with hematoxylin (blue; experiments were performed using organs from two unstimulated and four poly(I:C)-stimulated mice for each genotype with staining done at least in duplicate for each). Expression of TLR3 in the B cell area of the spleen was confirmed by staining with anti-B220 and anti-CD3 (not depicted). WP, white pulp; RP, red pulp. Bars, 100 µm.

MDA5$^{-/-}$ BMDCs also failed to produce adequate amounts of IFN-β, although a very minor IFN-β response was detectable early after poly(I:C) stimulation (Fig. 3 D). The IFN-β response was partially reduced in TLR3$^{-/-}$ BMDCs and completely abolished in DKO BMDCs. Thus, MDA5 is essential for both IFN-α and IFN-β responses to poly(I:C), whereas TLR3 is dispensable for IFN-α, although it contributes to IFN-β production. As opposed to type I IFNs, MDA5$^{-/-}$ BMDCs secreted WT levels of IL-12p40, whereas TLR3$^{-/-}$ BMDCs produced essentially no IL-12p40 (Fig. 3 E).

We conclude that MDA5 and TLR3 induce cytokines in response to poly(I:C) in different ways. MDA5 is required for the IFN-α and IFN-β response to poly(I:C), but not IL-12p40. In contrast, TLR3 is required for IL-12p40 and, to a certain extent, IFN-β, but is not essential for IFN-α production. Consistent with previous experiments in vivo (Longhi et al., 2009), we found that poly(I:C)-induced NK cell activation in vitro was severely impaired in the presence of an antibody that blocks the receptor for type I IFN (IFN-α/β receptor [IFNAR]; Fig. S2, A and B). Moreover, co-cultures of NK cells and DCs lacking IFNAR showed that NK cell activation requires IFN-α signaling in both NK cells and DCs (Fig. S2, C and D). Thus, type I IFNs are required for robust NK cell activation. Because MDA5$^{-/-}$ mice secrete very little IFN-α and IFN-β in response to poly(I:C), the scarcity of these cytokines is probably responsible for the global defect in NK cell activation in MDA5$^{-/-}$ mice after stimulation with poly(I:C). Although IL-12 stimulates IFN-γ secretion (Trinchieri, 1995),

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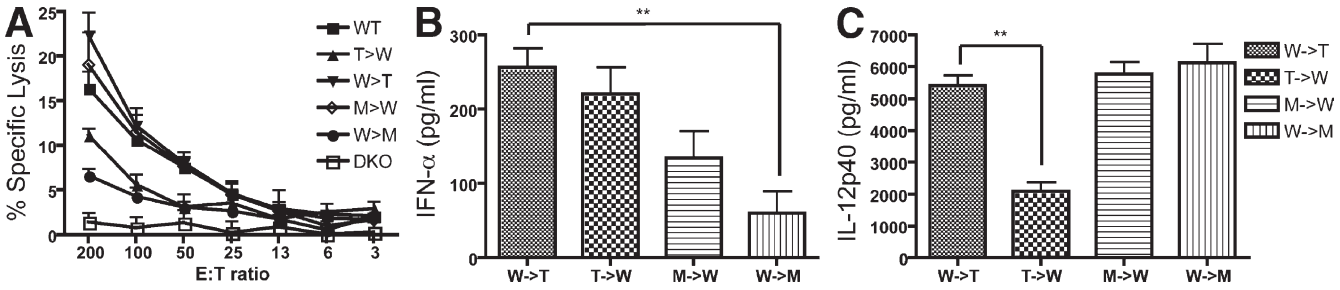

Figure 5. MDA5 and TLR3 act in different cellular compartments. BM chimeras consisting of WT>MDA5$^{-/-}$, MDA5$^{-/-}$>WT, WT>TLR3$^{-/-}$, and TLR3$^{-/-}$>WT mice were stimulated with 100 µg poly(I:C) i.v. After 24 h, splenocytes were harvested and used as effector cells in cytotoxicity assays against Cr^{51}-labeled RMA-S targets (experiments were performed using one mouse for each chimera in three independent trials; A). Additionally, serum was collected to measure systemic IFN-α (B) and IL-12p40 (C; experiments were performed with serum from four mice of each chimera in one independent ELISA assay). Statistical significance is indicated by **, $P < 0.001$. Error bars represent SD.

the addition of an antibody neutralizing IL-12 to co-cultures of NK cells and DCs had minimal impact on poly(I:C)-induced NK cell secretion of IFN-γ (Fig. S2, A and B). Thus, the partial defect in IFN-γ secretion observed in TLR3$^{-/-}$ mice may be caused by insufficient IFN-β, perhaps combined with the lack of IL-12 and/or other cytokines (Matikainen et al., 2001). As yet undefined TLR3-induced cell–cell interactions might also contribute to NK cell secretion of IFN-γ.

MDA5 and TLR3 function in different accessory cell populations

Our data indicate that MDA5 and TLR3 have distinct quantitative and qualitative impacts on NK cell activation by poly(I:C). One potential explanation for this is differential expression of MDA5 and TLR3 in cell types that have distinct capacities to produce cytokines. To address this possibility, we investigated the expression of MDA5 and TLR3 in the spleen and liver before and after poly(I:C) stimulation by immunohistochemistry. In naive mice, MDA5 was broadly expressed in the red pulp and T cell area of the spleen and in the hepatocytes and interstitial cells of the liver (Fig. 4 A). In contrast, TLR3 expression was more limited, including DCs of the white pulp, rare lymphoid cells in the marginal zone, red pulp macrophages, and liver interstitial cells, likely corresponding to Kupffer cells and endothelial lining cells (Fig. 4 B). Poly(I:C) stimulation induced a very strong increase in MDA5 expression in both spleen and liver, with the only notable exception in the splenic B cell area (Fig. 4 A). Poly(I:C) stimulation also induced broader expression of TLR3 in the spleen, particularly in the B cell area, and in the liver, including the hepatocytes (Fig. 4 B). These results suggested that MDA5 and TLR3 are constitutively expressed in partially distinct cellular compartments of the spleen and liver, with MDA5 being more broadly expressed than TLR3. Administration of poly(I:C) stimulates a stronger and broader expression of both sensors, consistent with previous studies showing that type I IFNs induce MDA5 (Ishii et al., 2008) and TLR3 expression (Matsumoto and Seya, 2008). Even under these conditions, however, the distribution of MDA5 and TLR3 does not entirely overlap.

To test the importance of MDA5 and TLR3 in the stromal versus hematopoietic compartments, we created radiation chimeras between WT and MDA5$^{-/-}$ as well as WT and TLR3$^{-/-}$ mice. Upon poly(I:C) stimulation, defective NK cell–mediated cytotoxicity and reduced serum levels of IFN-α were seen in MDA5$^{-/-}$ chimeras that had received WT BM, whereas no cytotoxicity defect and minor impairment of systemic IFN-α were observed in WT chimeras that had been grafted with MDA5$^{-/-}$ BM (Fig. 5, A and B). Conversely, WT hosts that had received TLR3$^{-/-}$ BM showed a slight decrease in cytotoxicity and marked reduction of systemic IL-12p40 compared with WT animals, whereas no obvious cytotoxicity or systemic IL-12p40 defects were observed in chimeras from TLR3$^{-/-}$ hosts grafted with WT BM (Fig. 5, A and C). These results indicate that MDA5 activates NK cells by acting predominantly in the radio-resistant stromal cell population, whereas TLR3 activates NK cells mainly through radio-sensitive hematopoietic accessory cells.

Poly(I:C)-mediated NK cell activation in vivo occurs independently of CD8α DCs

Because TLR3 is highly expressed in CD8α DCs (Edwards et al., 2003) and CD8α DCs specialize in the secretion of IL-12 (Maldonado-López et al., 1999), it seemed plausible that the hematopoietic accessory cells involved in TLR3-induced NK cell activation were, in fact, CD8α DCs. This possibility was further supported by a recent study showing that poly(I:C) triggers the TRIF and IPS1 signaling pathways in CD8α DCs, inducing the secretion of IL-12 and type I IFNs that activate NK cells in vitro (Miyake et al., 2009). To directly test the contribution of CD8α DCs to poly(I:C)-mediated NK cell activation in vivo, we analyzed Batf3$^{-/-}$ mice, which selectively lack the CD8α DC population (Hildner et al., 2008). After injection of poly(I:C), NK cells isolated from Batf3$^{-/-}$ killed RMA-S cells only slightly less efficiently than WT NK cells (Fig. 6 A). Up-regulation of CD69, NK cell secretion of IFN-γ, serum IFN-α, and serum IL-12p40 were similar in Batf3$^{-/-}$ and WT mice (Fig. 6, B–E). These results indicate that poly(I:C) triggers secretion of NK cell stimulatory cytokines through multiple accessory cells rather than solely through CD8α DCs.

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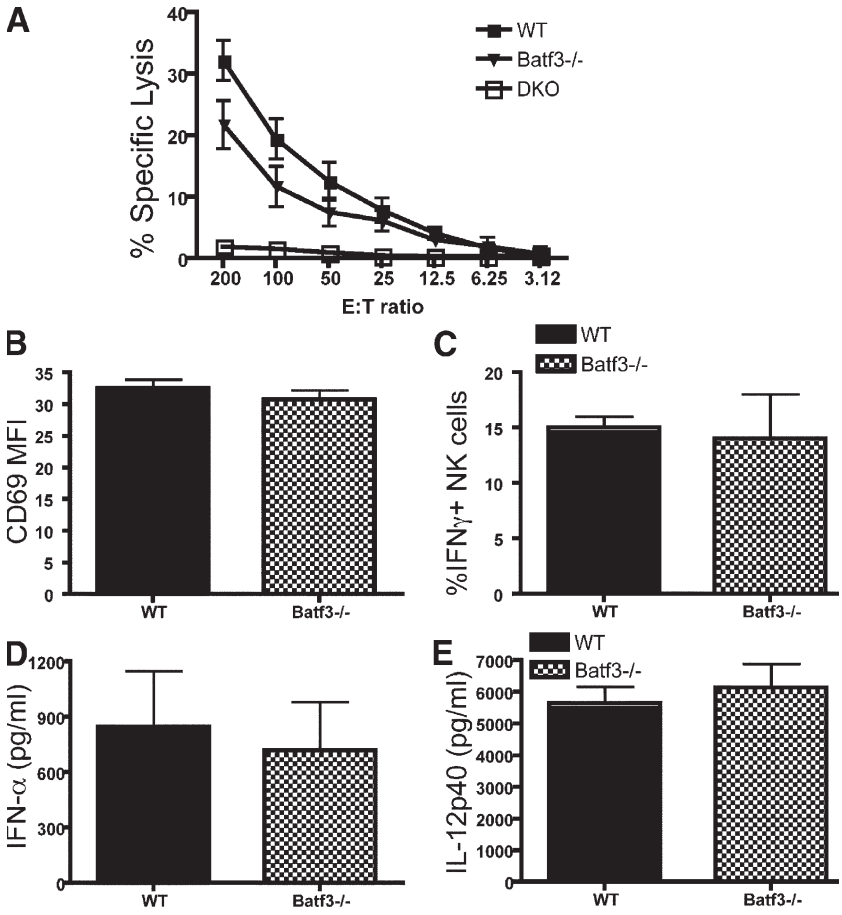

Figure 6. Poly (I:C)–induced NK cell activation is independent of CD8α DCs. WT and Batf3$^{-/-}$ mice were injected with 100 µg poly(I:C) i.v. After 24 h, splenocytes were harvested and used as effector cells in a cytotoxicity assay with labeled RMA-S targets (experiments were performed using one mouse for each genotype in three independent trials; A) or assayed for CD69 expression by FACS, gating on DX5^{+}CD3^{-} splenocytes (experiments were performed using two mice for each genotype in two independent trials; B). Alternatively, 3 h after poly(I:C) injection, splenocytes were isolated and cultured with monensin for an additional 3 h, at which time DX5^{+}CD3^{-} cells were analyzed by FACS for intracellular IFN-γ content (experiments were performed using one mouse for each genotype in three independent trials; C). Serum samples from poly(I:C)-injected mice were taken at 24 h, and IFN-α (D) and IL-12p40 (E) were measured by ELISA (experiments were performed with serum from four mice of each chimera in one independent ELISA assay). Error bars represent SD.

Concluding remarks

In this study, we provide the first demonstration that MDA5 is essential for robust activation of mouse NK cells in response to poly(I:C). Although previously published studies have suggested that poly(I:C) activates NK cells primarily through TLR3, our data show that MDA5 is, in fact, more important than TLR3 for triggering all NK cell functions, including cytotoxicity, CD69, and IFN-γ production. TLR3 has a minor impact on NK cell activation, and its role is most evident in DKO mice, in which the lack of MDA5 and TLR3 completely abrogates the NK cell response to poly(I:C). This result also excludes any contribution of other dsRNA sensors, such as RIG-I, to poly(I:C)-mediated NK cell activation.

We demonstrated that MDA5- and TLR3-mediated NK cell activation is NK cell extrinsic. This conclusion is supported by in vitro experiments showing that lack of both MDA5 and TLR3 in NK cells has no impact on the ability of poly(I:C) to induce NK cell activation in the presence of WT DCs, whereas deficiency of MDA5 and/or TLR3 in DCs impairs activation of WT NK cells. Similar results were recently obtained by Miyake et al. (2009). Moreover, the extrinsic function of MDA5 was further supported by in vivo experiments showing that poly(I:C)-mediated NK cell activation is normal in lethally irradiated WT mice reconstituted with MDA5$^{-/-}$ BM cells, which generate MDA5$^{-/-}$ NK cells. Although MDA5 is ubiquitously induced by type I IFNs and therefore may be also expressed in NK cells, NK cells most likely lack efficient mechanisms for poly(I:C) uptake, thereby preventing a direct effect of poly(I:C) on NK cells. It remains possible that the administration of poly(I:C) with liposomal reagents that facilitate cytosolic entry of poly(I:C) may induce some direct activation of NK cells.

MDA5 and TLR3 contributed to poly(I:C)-induced NK cell activation by inducing different NK cell stimulatory cytokines. MDA5 promoted IFN-α and IFN-β secretion, whereas TLR3 was essential for IL-12p40 and, in part, for

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IFN-β production. Our in vitro data indicate that type I IFNs are crucial for poly(I:C)-induced NK cell activation. These data are consistent with the recent observation that NK cell secretion of IFN-γ in response to poly(I:C) is blocked by injection of an anti-IFNAR antibody in vivo (Longhi et al., 2009). The crucial role of type I IFNs in poly(I:C)-mediated NK cell activation, together with the predominant function of MDA5 in inducing IFN-α and IFN-β secretion, explains the major defect in NK cell activation in MDA5$^{-/-}$ mice. Although IL-12 is a known inducer of IFN-γ (Trinchieri, 1995), blockade of IL-12 did not affect poly(I:C)-induced secretion of IFN-γ by NK cells, at least in vitro. Therefore, the transient defect in NK cell secretion of IFN-γ observed in TLR3$^{-/-}$ mice may be caused by insufficient IFN-β, perhaps combined with a defect in IL-12 and/or other cytokines (Matikainen et al., 2001). Although our data underscore the roles of MDA5 and TLR3 in inducing IFN-α and IL-12, MDA5 and TLR3 may also act by inducing cell surface molecules on accessory cells that activate NK cells through cell–cell interactions or local delivery of cytokines. This is the case for the α chain of the IL-15 receptor (IL-15Rα), which is induced by type I IFNs and allows accessory cells to trans-present IL-15 to NK cells (Koka et al., 2004; Lucas et al., 2007; Mortier et al., 2008). Accordingly, transcriptional analysis of WT, MDA5$^{-/-}$, and TLR3$^{-/-}$ BMDCs stimulated in vitro with poly(I:C) showed that both MDA5 and TLR3 are required for the induction of both IL-15Rα and its ligand IL-15 (Fig. S3). MDA5 and TLR3 may induce other cell surface molecules on accessory cells involved in NK cell activation, either indirectly through type I IFN signaling or directly through IPS1 and TRIF and their downstream transcription factors IRF1/3/7 and NF-κB.

Our data indicate that the disparate impacts of MDA5 and TLR3 on poly(I:C)-induced secretion of IFN-α, IFN-β, and IL-12p40 and NK cell activation may be related, at least in part, to the distinct cellular distribution of MDA5 and TLR3. BM chimera experiments demonstrated that MDA5 mainly acts through stromal cells. The surfeit of these cells and their general ability to produce type I IFNs can explain why MDA5 stimulation leads to a major release of IFN-α. MDA5 may have a more limited role in IL-12 secretion because, although IFN-α induces IL-12p35 (Hermann et al., 1998; Gautier et al., 2005), it also modulates IL-12 production from DCs and monocytes/macrophages (Nguyen et al., 2000). In contrast, TLR3 has a more restricted distribution and acts mainly through hematopoietic cells. This may explain why TLR3 has a minor impact on systemic IFN-α and a more prominent effect on IL-12 production. Although CD8α DCs express TLR3 (Edwards et al., 2003), specialize in the secretion of IL-12 (Maldonado-López et al., 1999), produce type I IFNs (Longhi et al., 2009), and strongly activate NK cells in vitro in response to poly(I:C) (Miyake et al., 2009), our analysis of BATF3$^{-/-}$ mice that lack CD8α DCs demonstrates that poly(I:C)-mediated NK cell activation occurs even in the absence of these cells, indicating that TLR3 acts through a variety of hematopoietic accessory cells. Immunohistochemical analysis of TLR3 expression suggests that these cells may include various DCs and macrophage subsets in the white and the red pulp of the spleen as well as marginal zone B cells. In conclusion, our experiments elucidate the mechanisms by which poly(I:C) activates NK cells in vivo, demonstrating distinct yet complementary roles for MDA5 and TLR3 in stimulating NK cell effector functions through a multiplicity of accessory cells.

MATERIALS AND METHODS

Mice, cell lines, antibodies, and poly(I:C). MDA5$^{-/-}$, TLR3$^{-/-}$, and IFNAR$^{-/-}$ mice have been described previously (Alexopoulou et al., 2001; Trumpfheller et al., 2008). DKO mice were made by intercrossing MDA5$^{-/-}$ and TLR3$^{-/-}$ mice. These mice have been backcrossed to the C57BL/6 background. Age- and sex-matched C57BL/6 control mice were purchased from Jackson ImmunoResearch Laboratories, Inc. Batf3$^{-/-}$ (Hildner et al., 2008) and WT (Taconic) mice were on the 129SvEv background except for those used for IFN-γ staining, which were backcrossed six times onto the C57BL/6 background. All mouse protocols were approved by the Washington University Animal Care Committee. RMA-S cells were maintained in RPMI 1640 supplemented with 10% FCS, penicillin/streptomycin, and glutamax. Blocking antibodies included anti-IFNAR (MAR1-5A3), anti–IL-12 (Tosh; provided by E.R. Unanue, Department of Pathology and Immunology, Washington University School of Medicine, St Louis, MO), and anti-human IFN-γ receptor (GIR-208) as isotype control. High molecular weight poly(I:C) was obtained from InvivoGen.

BM chimeras. Recipient mice were γ irradiated with 1,000 rad. After an overnight rest, mice were reconstituted with 5×10^6 BM cells per mouse that had been harvested from the femurs and tibias of age- and sex-matched donors. After 6 wk, chimeras were used for in vivo poly(I:C) stimulations.

Cell preparations. Single-cell suspensions were prepared from spleens and depleted of erythrocytes by ammonium chloride lysis. For NK purification, cell suspensions were incubated with anti-DX5–coated MACS beads (Miltenyi Biotec) and purified by autoMACS. Primary cells were cultured in complete media (RPMI 1640 without L-glutamine supplemented with 10% FCS, sodium pyruvate, kanamycin sulfate, glutamax, and nonessential amino acids). BMDCs were cultured in complete media with 2% GM-CSF for 7 d and used in assays with complete media.

NK-DC co-cultures. For NK-DC cell co-culture experiments, 10^5 BMDCs were cultured with 5×10^4 NK cells in the presence or absence of 25 μg poly(I:C). In some experiments, anti-IFNAR, anti–IL-12p70, or control antibody was added to the cultures before the addition of the poly(I:C). In other experiments, purified NK cells alone were stimulated with 0, 25, or 100 μg poly(I:C) in the presence of 200, 1,000, or 5,000 U IFN-α (PBL Interferon Source); 1, 10, or 100 ng/ml IL-12 (PeproTech); or 1 ng/ml each of IL-12 and IL-18 (PeproTech). After 24 h, supernatants were harvested for cytokine detection, and NK cells were detached by washing with 1 mM EDTA in PBS and were analyzed by FACS.

Cytotoxicity assays. To measure NK cytotoxicity ex vivo, splenocytes were prepared as described previously (see Cell preparations section) 24 h after injecting mice with 100 μg poly(I:C) i.v. and were mixed with 10^5 Cr^{51}-labeled RMA-S targets in decreasing effector/target ratios. To measure NK cytotoxicity in NK-DC co-cultures, Cr^{51}-labeled RMA-S targets were directly added to the NK-DC co-cultures. After 4 h, supernatants were harvested, and Cr^{51} release was measured in individual samples as well as maximum and spontaneous release samples. Specific lysis was calculated by specific release − spontaneous release/max release − spontaneous release.

FACS analysis. Splenocytes, cultured NK cells, and BMDCs prepared as described previously (see Cell preparations section) were treated with Fc block (HB-197) and stained with anti-CD3, anti-NK1.1, and anti-CD69

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(BD) for NK cell activation experiments or with anti-DX5 in place of NK1.1 for Batf3$^{-/-}$ mice. Samples were processed on a FACSCalibur and analyzed with CellQuest software (BD).

Ex vivo IFN-γ production. Mice were injected with 100 μg poly(I:C) i.v. After 3 or 4 h, splenocytes were prepared as described previously (see Cell preparations section) and cultured with monensin for an additional 3 or 4 h. After incubation, cells were stained with anti-CD3 and anti-NK1.1 or anti-DX5 (Batf3$^{-/-}$), fixed with paraformaldehyde, permeabilized with saponin buffer, and stained with anti–IFN-γ. Samples were then analyzed by FACS as described previously (see FACS analysis section) to detect the percentage of IFN-γ–producing NK cells.

Cytokine measurements. Serum samples were taken at 6 and 24 h after injecting mice with 100 μg poly(I:C); supernatants of NK-DC cultures were harvested at 0, 6, 12, and 24 h after poly(I:C) stimulation. Type I IFNs and IL-12p40 were determined by ELISA (PBL Interferon Source and eBioscience, respectively); IFN-γ was assessed by cytometric bead array (BD).

Statistics. Figures were plotted using Prism4 (GraphPad Software, Inc.), indicating the mean and SD. Statistical significance was determined primarily by Student's *t* test. Analysis of variance was used to determine significance for cytotoxicity assays. Significance is indicated by *, $P < 0.05$, **, $P < 0.001$, and ***, $P < 0.0001$.

Immunohistochemistry. Spleen and liver sections were obtained from frozen (for anti-TLR3 staining) and formalin-fixed paraffin-embedded tissue (for anti-MDA5 staining). Primary antibodies included anti-MDA5 (rabbit polyclonal anti–mouse; AL180; Enzo Life Sciences, Inc.), anti-TLR3 (rat anti–mouse 11F8.1B7; provided by D.M. Segal, Experimental Immunology Branch, National Cancer Institute, Bethesda, MD), anti-B220 (Invitrogen), and anti-CD3 (rabbit monoclonal SP7; Thermo Fisher Scientific). Anti-TLR3 and -B220 were detected after endogenous biotin blocking using a rabbit anti–rat IgG (mouse absorbed; Vector Laboratories). For anti-MDA5 and -CD3 stainings, sections were deparaffinized and subjected to antigen retrieval by incubating in a water bath at 98°C for 40 min. Primary antibodies were detected using Envision rabbit (Dako). Reactions were revealed by diaminobenzidine.

RNA preparation and RT-PCR. BMDCs were stimulated with 25 μg poly(I:C) for 6 or 12 h, and then RNA was harvested from the cells using an RNeasy kit (Invitrogen). cDNA was synthesized from RNA (Superscipt RT kit; Invitrogen), and relative levels of IL-15 and IL-15Ra were determined by semiquantitative PCR and normalized to GAPDH using the following primers: IL-15 sense 5′-GCAGAGTTGGACGAAGAC-3′, IL-15 antisense 5′-AGCACGAGATGGATGTATT-3′, IL-15Rα sense 5′-TCTCCCCACAGTTCCAAAAT-3′, IL15Rα antisense 5′-GGCACCCAGGCTCAGTAAAA-3′, GAPDH sense 5′-GAGCCAAAAGGGTCATCATC-3′, and GAPDH antisense 5′-CCATCCACAGTCTTCTGGGT-3′.

Online supplemental material. Fig. S1 shows the limited effect of poly(I:C) on purified NK cells. Fig. S2 shows that type I IFN is essential for poly(I:C)-induced NK cell activation through NK cell–intrinsic and –extrinsic mechanisms. Fig. S3 shows that MDA5 and TLR3 are required for poly(I:C)-induced expression of IL-15Rα and IL-15. Online supplemental material is available at http://www.jem.org/cgi/content/full/jem.20091181/DC1.

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